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(54) Title: TUMOR CELL BINDING MOLECULE WITH PROTEASE INHIBITING REGION

(57) Abstract

A molecule comprising a first region which binds to a tumour (preferably the receptor for uPA) and a second region which inhibits a (preferably tumour-associated) protease, for example uPA. The first and second regions may be respective antibodies or parts thereof specific for appropriate parts of uPAR and uPA. Preferably, the first region is a uPAR-binding part of uPA, e.g. the 12-32 region theeof, and the second region is PAI-2 or a uPA-inhibiting analogue or part thereof, and the first and second regions are combined to form a single polypeptide, expressible from a transformed host.

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TUMOR CELL BINDING MOLECULE WITH PROTEASE INHIBITING REGION

The process of activation of the zymogen plasminogen to the broad spectrum serine protease, plasmin, is mediated by specific plasminogen activators: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA is primarily responsible for the generation of plasmin in fibrinolysis and its activity is stimulated when bound to fibrin. In humans, and many other species, uPA is important in generating proteolytic activity responsible for extracellular matrix and basement membrane degradation associated with tissue remodelling. Such processes are involved in growth and spread of tumour cells, mammary gland involution, ovulation, embryo development, development of the nervous system, in the normal inflammatory response and in a number of inflammatory diseases (Danø *et al*, 1985). Elevated levels of uPA have been found in malignant tissue and there is believed to be a general correlation between the amount of uPA and the invasiveness of the tumour. Studies of experimental Lewis lung tumours revealed concentration of uPA at the invasive edge of the tumours. In addition, anti-catalytic antibodies to uPA inhibited the establishment of tumour cells after i.v. injection into mice and inhibited metastasis in chick embryos. The proteolytic activity released by uPA is localized by the binding of plasminogen, plasmin and uPA to specific cell surface receptors (Vassalli *et al*, 1985; Miles and Plow, 1988) and the importance of receptor-bound uPA in tissue invasion has been demonstrated (Ossowski, 1988). The receptor-binding region has been localized to the growth factor domain of the uPA

molecule in the region of amino acids 12-32 of mature uPA since peptides corresponding to this sequence are able to block the uPA receptor interaction by competition (Appella *et al*, 1987).

The apparent central role of uPA in malignant disease indicates that inhibition of the activity of uPA might modify the course of the disease. Of the natural inhibitors of uPA, only plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2) are sufficiently active and specific enough to be considered (Sprengers and Kluft, 1987). Although PAI-1 is a faster inhibitor of uPA than PAI-2 it is also a very effective inhibitor of tPA and administration of this protein would be likely to compromise fibrinolysis. PAI-2, on the other hand, is a slower inhibitor of tPA and is reported to be inactive against the physiologically important fibrin-stimulated tPA. PAI-2 has been shown to inhibit receptor-bound uPA (Kirchheimer and Remold, 1989a; Pöllänen *et al*, 1990) and uPA-dependent tissue invasion (Kirchheimer and Remold, 1989b) and thus represents a potentially useful anti-tumour or anti-metastatic agent. Natural sources of PAI-2 yield very small quantities of the protein but EP-A-238 275 discloses the production of recombinant PAI-2 in *E. coli*. Our co-pending application GB8918191.1 discloses the advantageous production of recombinant PAI-2 in *Saccharomyces cerevisiae*.

Fusions of PAI-2 and other polypeptides are described in EP-A-238 275 (page 30) but only in the context of obtaining secretion of the PAI-2 from a transformed cell producing it or

in the context of detecting PAI-2 production as a ß-galactosidase fusion.

WO 88/08451 discloses uPA-tPA fusions, but the portion of uPA which is used is the B chain and the intention is to retain the activities of both tPA and uPA. As is described below, the uPA-derived portion (if present) of the molecules of the present invention preferably has substantially no uPA-like amidolytic or proteolytic activity.

One aspect of the invention provides a molecule comprising a first region which binds to a tumour cell and a second region which inhibits a first protease.

The specific tumour-binding may be enabled by the existence on the tumour of structures unique to the tumour, for example tumour-specific antigens such as CEA (carcino-embryonic antigen), pan carcinoma antigen, placental alkaline antigen or polymorphic epithelial mucin antigen. Alternatively, the specificity may arise from the existence of a higher level or density on the tumour cell surface of a structure which is found on normal cells; the receptor for uPA (uPAR) is an example of such a structure.

The tumour-binding region is preferably an entity which will bind to the receptor for uPA (uPAR), as is described in more detail below. Alternatively, it may be any other entity which binds preferentially to a tumour cell, for example the epidermal

growth factor receptor present in some breast carcinomas.

The first protease is preferably tumour-associated and is preferably urokinase-type plasminogen activator (uPA) as is described in more detail below. However, it could be any other protease of which high local levels are associated with tumours, or at least tumours of a given kind. Preferably the protease is one that can be activated or converted from a pro-enzyme or zymogen form by uPA or receptor-bound uPA or by plasmin to generate localised proteolytic activity. The inhibitor region in the molecule of the invention may be an inhibitor of plasmin such as the Pittsburgh variant of α_1-antitrypsin, an α_1AT variant having lysine at the P_1 position, α_2-antiplasmin, α_2-macroglobulin, aprotinin or any other inhibitor with plasmin inhibitory activity, or it may be an inhibitor of a collagenase such as tissue inhibitor of metallo-proteinases (TIMP) or the related TIMP2. Other proteases believed to be involved in tissue remodelling in neoplasia are proteases of the cathepsin and stromelysin families and it may be beneficial to provide an inhibitor of these activities. Another protease (not tumour-associated) is thrombin, a potent activator of platelet aggregation which activates a number of other cell types, including endothelial cells, thus initiating their response to stress. This serine protease is consequently an important regulator of thrombosis and haemostasis in addition to its role in generation of fibrin. The action of thrombin on platelets and endothelial cells is mediated by the thrombin receptor (Vu *et al*, 1991) and activation of this receptor involves the cleavage of

an extracellular domain of the receptor by thrombin. Thus, the activation of the thrombin receptor and the consequent activation of platelets and other cell types may be inhibited by a thrombin inhibitor such as α_1-antitrypsin Pittsburgh. An application of the concept of the current invention to this system is to enhance the anti-thrombin-receptor-activating activity of thrombin by directing the α_1-AT Pittsburgh to the thrombin receptor or an adjacent cell surface molecule. In this instance the receptor-binding moiety of the hybrid may be a receptor-binding peptide or an antibody or antibody fragment directed against the thrombin receptor. In all of these cases an inhibitory portion of the protease inhibitor may be used instead of the whole molecule.

The molecule thus provides for the targeting of the protease inhibiting region to the tumour cell.

The protease the activity of which is inhibited may be free, bound to its receptor, associated with the cell surface via non-specific interactions or bound to adjacent cells or to extracellular matrix.

The first region is preferably a peptide sequence corresponding to a receptor-binding a region of the tumour-associated protease, which may or may not be the same protease as the protease which is inhibited. Thus, the first region may comprise amino acids 1-32 of uPA or a variant or fragment thereof. By "variant" we mean a region with one or more minor alterations to the amino acid sequence thereof which region is

nevertheless recognisably similar to the said 1-32 region and which still binds to the uPA-binding site on the uPAR. Incidentally, although the region is generally "EGF-like", epidermal growth factor (EGF) itself does not bind to the said site. By "fragment" we mean smaller sequences (preferably at least 10, 15, 19 or 20 amino acids long) which still bind to the binding site of the receptor, for example the 20-30 region of uPA. "Variants" of "fragments" may also be used. The receptor-binding region of the protease may, in the molecule of the invention, be accompanied by other parts of the protease or a pro-form of the protease or a mutant thereof. Such parts or mutants should have at least 50% homology, preferably 60%, 70%, 80%, 90% or 95% homology with the protease (or, in the case of parts, with the corresponding region of the protease) in order to be regarded as parts or mutants thereof. They should, however, at least when present in the molecule of the invention, not have any undesirable level of protease-like proteolytic activity, since otherwise the point of the invention would be lost. Preferably, the protease-derived part of the molecule of the invention, at least when present in the said molecule, has substantially no protease-like proteolytic activity. In the case of uPA, it may be possible to achieve this simply by using a pro-uPA mutant in which Lys^{158} has been altered, for example to Gln or Gly, to prevent cleavage by plasmin. The 12-19 and 31-32 residue regions adjacent the 20-30 binding region of uPA seem to help the binding region to bind optimally and are preferably present, or functionally equivalent flanking regions are present.

The first region may alternatively be a mon clonal antibody or part thereof or a genetically engineered counterpart thereof which binds to the protease receptor, preferably uPAR. Preferably, it binds to the protease-binding site of the receptor or it binds to an area of the receptor which is sufficiently close to the said binding site for the bulk of the molecule as a whole to block binding of the protease to the receptor. In this way, not only is the protease-inhibiting region targeted to the receptor, but the protease-binding site of the receptor is blocked, which will prevent binding of the protease to that receptor and thus may further help to prevent the protease from acting on its substrate. Monoclonal antibodies to the protease receptor or other target entity may be prepared by immunising an animal with the target entity or an antigenic part thereof and preparing and selecting an appropriate hybridoma in the conventional way (see Monoclonal Hybridoma Antibodies Ed. J.G.R. Hurrell, CRC Press 1982, incorporated herein by reference). Alternatively, a polyclonal collection of antibodies of restricted specificity may be used. Preferably, however, the antibody is a monoclonal antibody, an F_{ab}', $(F_{ab})_2$, F_v, scF_v or dAb fragment or a synthetic molecule including the minimum recognition unit(s) of a target-entity-specific immunoglobulin.

In other embodiments, the first region consists of any peptide which will bind to the protease-binding site, or sufficiently close to it to block it, and need not be homologous with any part of the protease.

The second region may be any compound which inhibits the action of the protease (preferably uPA). Such a compound may be an antibody (or antibody fragment etc, as discussed above) directed against the enzymatically active site of the protease, or against such a part of the protease molecule as to block access of the substrate to the active site or against any other part of the protease such that binding of the antibody to the said part prevents or at least reduces the activity of the protease towards its substrate.

In a particularly preferred embodiment, however, the second region is PAI-2 or part thereof or a mutant PAI-2 or part thereof. Preferably, the mutant or part of PAI-2 retains a useful level of the uPA-inhibiting activity of PAI-2. Fusions of the uPAR-binding domain of uPA and PAI-2 or a part or mutant thereof (hereinafter called "uPA-PAI-2 fusions") may exhibit the following properties:

1. binding unoccupied uPAR (ie uPAR unoccupied by uPA) via the growth factor domain, thereby blocking binding by uPA to uPAR,

2. in solution, inhibiting free or receptor-bound uPA, and

3. when receptor-bound, inhibiting receptor-bound uPA.

The molecule should be stable *in vivo*. By "stable *in vivo*", we mean that the molecule is sufficiently stable when

administered parenterally, preferably intravenously, to allow useful quantities of it to reach a tumour and bind to the receptors on the tumour cells. In practice, a molecule with a half-life in the human circulation of 30 minutes or longer is considered to be stable. Preferably, the half-life is at least 1 hour, 24 hours, 3 days or more.

The molecules of the invention may be dissolved in suitable delivery vehicles and administered to patients to inhibit or prevent the growth or spread of an actual or suspected tumour. The patient is preferably a human but may be another animal, preferably a mammal, such as a pet (dogs, cats, etc) or an economically important animal (sheep, cattle, pigs, fowl, horses, etc). In the case of administration to animals, the tumour-binding domain is modified for optimal binding to the animal receptor and ideally is the sequence of the said domain of the protease of that animal. In addition, the inhibitor moiety may be modified to effect efficient inhibition of the protease of that animal. The molecules are preferably administered parenterally, for example intravenously, intramuscularly or subcutaneously, by injection or infusion. Clinically qualified people will be able to determine suitable dosages, delivery vehicles and administrative routes.

The molecules of the invention may be produced by chemically linking the said first and second regions by methods known t those in the art of protein chemistry, for example using the methods of O'Sullivan *et al* (1979) and bifunctional linking

reagents such as m-maleimido-benzoyl-N-hydroxy-succinimide ester. However, it is preferred for the molecules to be produced by expression of a recombinant DNA sequence in a suitable host transformed therewith. Such hosts are legion and include *E. coli*, *B. subtilis*, Aspergillus and other filamentous fungi, yeasts, animal cells, plant cells, insect cells and transgenic whole animals and plants.

Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschunikowia*, *Rhodosporidium*, *Leucosporidium*, *Botryoascus*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*, because the ability to manipulate the DNA of these yeasts has, at present, been more highly developed than for the other genera mentioned above.

Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*.

Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*.

Examples of *Hansenula* are *Hansenula polymorpha*, *Hansenula anomala* and *Hansenula capsulata*.

Yarrowia lipolytica is an example of a suitable *Yarrowia* species.

Yeast cells can be transformed by: (a) digestion of the cell walls to produce spheroplasts; (b) mixing the spheroplasts with transforming DNA (derived from a variety of sources and containing both native and non-native DNA sequences); and (c) regenerating the transformed cells. The regenerated cells are then screened for the incorporation of the transforming DNA.

It has been demonstrated that yeast cells of the genera *Pichia, Saccharomyces, Kluyveromyces, Yarrowia* and *Hansenula* can be transformed by enzymatic digestion of the cell walls to give spheroplasts; the spheroplasts are then mixed with the transforming DNA and incubated in the presence of calcium ions and polyethylene glycol, then transformed spheroplasts are regenerated in regeneration medium.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

In the case of PAI-2-based fusions, the protein is preferably produced as a soluble intracellular protein.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1, PHO5, TRP1, ADH1, ADH2*, the genes for glyceraldehyde-3-phosphate

dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, and hybrid promoters involving hybrids of parts of 5' regulatory regions with parts of 5' regulatory regions of other promoters or with upstream activation sites (eg the promoter of EPA-258067). The preferred promoter is the *PRB1* promoter.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

Suitable secretion leader sequences, if the molecule is to be secreted from the host, include mammalian leader sequences, such as the HSA and pro-uPA leader sequences, *S. cerevisiae* leader sequences such as the α-mating factor pheromone pre- and prepro- sequence, the invertase (*SUC2*) leader sequence, the *PHO5* leader sequence, or hybrid leader sequences such as the leader sequence of WO 90/01063.

The amino acid sequence of the N-terminal portion of uPA encompassing the gr wth factor domain is as follows:

13

10 20 30

S N E L H Q V P S N C D C L N G G T C V S N K Y F S N I H W C N

40 50

C P K K F G G Q H C E I D K S K T C

The sequence of residues 20-30 is believed to be responsible for the specificity of binding to the receptor and this sequence may represent the minimum requirement for binding to the receptor. Sequences adjacent to this region provide the proper conformation for optimum binding but may be substituted by a functionally equivalent structure. The uPA sequence incorporated into the fusion molecules preferably includes residues 12-32 and in the preferred embodiment includes residues 1-47 to ensure optimum binding to uPAR. It is not necessary for the first region of the molecule of the invention to be at or adjacent the N-terminal of the molecule. The second region can be N-terminal to the first region.

DNA sequences for expression of the molecules of the invention may be prepared by known techniques, for example by fusing cDNA fragments prepared from mRNAs corresponding respectively to the receptor-binding region of the protease and to a protease inhibitor or a part thereof.

Monoclonal antibodies may be prepared generally by the techniques of Zola (1988) which is incorporated herein by reference. Useful antigens for preparing either monoclonal or polyclonal antibodies are (for the second region) uPA, low

molecular weight uPA or the heavy (B) chain of uPA, and (for the first region) uPAR or peptides based on the uPA-binding domain thereof. Antibody fragments such as F_{ab} fragments, may be prepared therefrom in known ways. The antibodies may be humanized in known ways. Antibody-like molecules may be prepared using the recombinant DNA techniques of WO 84/03712.

The art of "antibody engineering" is advancing rapidly, as is described in Tan & Morrison (1988), Williams (1988) and Neuberger *et al* (1988) (all of which are incorporated herein by reference), and is well suited to preparing the first or second regions of the molecules of the invention or the whole molecule itself. Thus, bispecific antibodies, specific for an appropriate receptor and an appropriate site on the protease, may be made by any of the methods described by Williams (1988). Such molecules will block the protease-binding site of the receptor and also inhibit the protease. The antibody may alternatively be bispecific for the receptor and the protease inhibitor (or a mutant or fragment thereof) so that the antibody acts to bind the inhibitor to the receptor. The combination of the antibody bound to the inhibitor (or a mutant or fragment thereof) thus constitutes a molecule of the invention, whereas the antibody itself constitutes a precursor thereof, forming a separate aspect of the invention. The antibody specific for the receptor and the inhibitor may be combined with the inhibitor before being administered or may be co-administered therewith. By "co-administered", we d not necessarily mean that the antibody and the inhibitor must be administered simultaneously; it is

sufficient for them to combine in a therapeutically useful way in the body.

Chimaeric antibodies, where the F_c region is a human immunoglobin or part thereof, may be desirable for long term treatment, to reduce adverse immunological responses. Single chain antibodies may be used for either or both of the two regions of the molecules of the invention.

Preferred embodiments will now be described by way of example and with reference to the accompanying figures.

Figure 1 illustrates the construction of plasmids pDBP1, pDBP2 and pDBP3. E = *Eco*RI, P = *Pst*I, Bg = *Bgl*II, H = *Hin*dIII, B = *Bam*HI and A = *Afl*II. Only the *Afl*II site present in the PAI-2 encoding sequence is shown;

Figure 2 (on four sheets) shows a DNA sequence encoding, and corresponding amino acid sequence of, PAI-2 in pDBP1;

Figure 3 shows the DNA sequence of the *PRB1* promoter;

Figures 4 to 8 respectively illustrate plasmids pAYE333, pAYE334, pAYE335, pDBP5 and pDBP6;

Figure 9 illustrates the construction of plasmids pDJB117 and pDJB118;

Figure 10 illustrates the construction of plasmid pDBUP1;

Figure 11 shows flow cytometer histograms of U937 cells to detect binding of the uPA-PAI-2 hybrid protein to cells. Control cells are shown by the left hand peak and cells incubated with the hybrid protein are shown by the right hand peak. Detection of bound hybrid protein was with anti-PAI-2 antibody and FITC-conjugated secondary antibody;

Figure 12 illustrates the construction of plasmids pDBA2, pDBUA1, pDBUA2 and pDBUA3.

Figure 13 shows the DNA sequences and amino acid sequence encoded thereby, for $\alpha_1$antitrypsin in pDBA1.

Example 1

Standard recombinant DNA procedures are as described by Sambrook *et al*, 1989 unless otherwise stated.

General

A plasminogen activator inhibitor 2 coding sequence (Figure 1) may be derived from EP-A-238 275. The DNA may be obtained from *E. coli* deposited as ATCC 53585 in connection with EP 238 275. This contains the plasmid pBTA438, comprising the PAI-2 sequence. The sequences encoding the uPA growth factor domain may be assembled from synthetic oligonucleotides by standard

procedures.

However, we obtained a cDNA sequence for PAI-2 ourselves by analogous techniques. An expression vector was constructed in which DNA encoding amino acids 1-47 of uPA, preceded by a methionine initiation codon, directly preceded DNA encoding PAI-2 such that the two coding sequences were in frame. Expression of these sequences was under the control of the *S. cerevisiae PRB1* gene promoter and transcription termination was effected by the *S. cerevisiae ADH1* terminator.

Specific Detail

PAI-2 Coding Sequence

A lambda gt11 cDNA library constructed from mRNA isolated from 4-phorbol-12-myristate-13-acetate stimulated cells of the human monocyte-like histiocytic lymphoma cell line U937 (obtained from Clontech Laboratories Inc) was used as a source of PAI-2 cDNA. The library was screened using radioactively labelled oligonucleotide probes corresponding to the DNA sequences encoding the N-terminus (oligo 1) and C-terminal end (amino acids 400-410) (oligo 2) of the PAI-2 protein, respectively by standard procedures.

Oligo 1

5'-ATG GAG GAT CTT TGT GTG GCA AAC ACA CTC TTT-3

Oligo 2

5'-GCC GAA AAA TAA AAT GCA CTT GGT TAT CTT ATG-3'

From the putative positive clones we selected one clone (lambda gt11-186) which appeared to contain the entire PAI-2 coding region. This was confirmed by sequence analysis of the DNA insert in this clone following transfer to M13mp19 to form pDBP1 (Figure 1). The sequence is shown in Figure 2.

To facilitate insertion into expression vectors, restriction enzyme recognition sites were created at the 5' and 3' ends of the PAI-2 gene. A *Bgl*II site was created at the 5' end of the gene using the anti-sense oligonucleotide primer 1:

5'-TGCCACACAAAGATCTTCCATTGTTTCAATCT-3'

to create a mutation in the third position of the second codon as shown below:

```
                    M   E   D   L   C   V   A
5'...AGATTGAAACA ATG GAG GAT CTT TGT GTG GCA...3'
3'...TCTAACTTTGT TAC CTC CTA GAA ACA CAC CGT...5'
```

changed to:-

```
                    M   E   D   L   C   V   A
5'...AGATTGAAACA ATG GAA GAT CTT TGT GTG GCA...3'
3'...TCTAACTTTGT TAC CTT CTA GAA ACA CAC GCT...5'
                    └──────┘
                    BglII
```

An *Afl*II site was created at the 3' end of the gene using

the anti-sense olig nucleotide primer 2:

5'-CAGAAGCAGCACGCTTAGTCTTAAGGTGAGGAAATCTGCC-3'

to create mutations in the third position of the last codon (proline) and in the first base after the stop codon as shown below:-

```
      G   R   F   S   S   P  STOP
5'...GGC AGA TTT TCC TCA CCC TAA AACTAAGCGTGCTGCTTCTG...3'
3'...CCG TCT AAA AGG AGT GGG ATT TTGATTCGCACGACGAAGAC...5'
```

changed to:

```
      G   R   F   S   S   P   STOP
5'...GGC AGA TTT TCC TCA CCT TAA GACTAAGCGTGCTGCTTCTG...3'
3'...CCG TCT AAA AGG AGT GGA ATT CTGATTCGCACGACGAAGAC...5'
                          └─────┘
                          AflII
```

These two oligonucleotides were annealed to single stranded pDBP1 and then used in an *in vitro* mutagenesis procedure (oligonucleotide-directed *in vitro* mutagenesis system-version 2, Amersham plc) carried out according to the manufacturer's recommendations. A clone derived from this procedure and with the correct changes was designated pDBP2 (Figure 1).

Oligonucleotide linkers were then used to position restriction sites at either end of the gene which are suitable for insertion of the gene into an expression vector. The linker positioned at the 5' end of the gene was

Linker 1

5'-AGCTTGTCGACGGATCCAAAAAG ATG GAA

ACAGCTGCCTAGGTTTTTC TAC CTT CTAG-5'

*Hin*dIII *Bam*HI *Bgl*II

and the 3' linker was

Linker 2

*Hin*dIII

5'-TTAAGTCGACAAGCTTG

CAGCTGTTCGAACCTAG-5'

*Afl*II *Bam*HI

These two linkers were ligated with the *Bgl*II-*Afl*II PAI-2 gene fragment from pDBP2 into *Hin*dIII + *Bam*HI digested M13mp19 to form pDBP3 (Fig 1).

PRB1 Promoter

The structural gene, *PRB1*, for the *Saccharomyces cerevisiae* vacuolar endoprotease B has been isolated (Moehle *et al*, 1987a) on two *prb1* complementing plasmids called MK4 and FP8. When the yeast *Saccharomyces cerevisiae* is grown on glucose in shake flask culture, very little protease B activity is detected until the cells have catabolised the glucose and are utilising the ethanol

accumulated during growth (Saheki and Holzer, 1975; Jones *et al*, 1986). This is believed to be a consequence of a transcriptional control mechanism which represses mRNA accumulation until the glucose has been exhausted and the culture enters the diauxic plateau (Moehle *et al*, 1987a). Studies with protease B (*prb1*$^-$) deficient mutants implicate protease B in the protein degradation that occurs when negative cells are starved of nitrogen and carbon (Wolf and Ehmann, 1979; Zubenko and Jones, 1981).

The DNA sequence of the *PRB1* gene has been reported, as has 150bp of the *PRB1* promoter (Moehle *et al*, 1987b). A more extensive DNA sequence of the *PRB1* promoter is also available as an entry in the Genbank database, release 60, accession number M18097, locus YSCPRB1, Figure 3.

The whole of the *PRB1* promoter may be used, or a smaller portion thereof, as may readily be determined. For example, the roughly 1kbp sequence extending upstream from the start codon to the *Sna*B1 site is effective.

The 1.435kbp *Hin*dIII-*Eco*RI DNA fragment containing the protease B promoter (Figure 3) was cloned into the polylinker of the M13 bacteriophage mp18 (Yanisch-Perron *et al*, 1985), generating plasmid pAYE333 (Figure 4). Double stranded pAYE333 was linearised by partial digestion with *Sna*B1 and the double stranded oligonucleotide linker 3 inserted by ligation.

Linker 3

5'-GCGGCCGC-3'

3'-CGCCGGCG-5'

*Not*I

This generated a *Not*I restriction site at the 5' end of the protease B promoter. The promoter element was further modified by site directed mutagenesis (oligonucleotide direct *in vitro* mutagenesis system-Version 2, Amersham) according to the manufacturer's instructions. Mutagenesis with the oligonucleotide

5'-CGCCAATAAAAAAACAAGCTTAACCTAATTC-3'

introduced a *Hind*III restriction site close to the ATG translation initiation codon:

CGCCAATAAAAAAACAAACTAAACCTAATTCTAACAAGCAAAGATG

Met

CGCCAATAAAAAAACAAGCTTAACCTAATTCTAACAAGCAAAGATG

Met

*Hind*III

Plasmid pAAH5 (Goodey *et al*, 1987) was linearised by partially digesting with *Bam*HI. The 5' protruding ends were

blunt-ended with T4 DNA polymerase and ligated with the double-stranded oligonucleotide Linker 3. A recombinant plasmid pAYE334 (Figure 5) was selected in which a *Not*I restriction site had replaced the *Bam*HI site at the 3' end of the *ADH*1 terminator.

The 0.8kbp *Not*I-*Hin*dIII modified protease B promoter sequence was placed upstream of the 0.45kbp *Hin*dIII-*Not*I *ADH*1 transcription terminator on a pAT153-based plasmid (Twigg and Sherratt, 1980) to generate pAYE335 (Figure 6).

The large 6.38kbp *Hin*dIII-*Bam*HI fragment from the yeast *E. coli* shuttle vector pJDB207 (Beggs, 1981) was treated with the Klenow fragment of *E. coli* DNA polymerase to create flush ends and ligated with the double stranded olignonucleotide Linker 3 to generate plasmid pDBP5 (Figure 7).

The 1.25 kbp *Not*I Protease B promoter/*ADH*1 terminator cassette from plasmid pAYE335 (Figure 6) was introduced into the unique *Not*I site of plasmid pDBP5, generating pDBP6 (Figure 8).

uPA-PAI-2 fused coding region

Two oligonucleotides were synthesised using an Applied Biosystems Inc 380B oligonucleotide synthesiser and annealed to form a linker (linker 5) to encode the C-terminal portion of the uPA growth factor domain (amino acids 24-47 of mature uPA) and the start of the PAI-2 sequence.

Linker 5

```
     Y   F   S   N   I   H   W   C   N   C   P   K   K   F   G
TCGAGTAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA
    CATG AAG AGG TTC TAA GTG ACC ACG TTG ACG GGT TTC TTT AAG CCT
    └─────┘
    ScaI

 G   Q   H   C   E   I   D   K   S   M   E
GGG CAG CAC TGT GAA ATA GAT AAG TCA ATG GAA
CCC GTC GTG ACA CTT TAT CTA TTC AGT TAC CTT CTA G
                                        └─────┘
                                        BglII
```

This linker was ligated with a *Bgl*II-*Hin*dIII fragment of pDBP3, representing the remainder of the PAI-2 sequence, into pUC18 at *Sal*I-*Hin*dIII to form the plasmid pDJB117 (Figure 9).

A second linker representing the *PRB1* ATG environment, a methionine initiation codon and DNA encoding the N-terminal portion of the uPA growth domain (amino acids 1-23 of mature uPA) was assembled by annealing the four oligonucleotides shown below:-

Linker 6

oligo 3

```
                                        M   S   N   E   L   H   Q   V
GATCAAGCTTAACCTAATTCTAACAAGCAAAG ATG AGC AAT GAA CTT CAT CAG GTA
    TTCGAATTGGATTAAGATTGTTCGTTTC TAC TCG TTA CTT GAA GTA GTC CAT
    └────┘
    HindIII
    └──────────────────────────────────────────────────────────────
```

oligo 6

oligo 4

```
 P   S   N   C   D   C   L   N   G   G   T   C   V   S   N   K
CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG
GGT AGC TTG ACA CTG ACA GAT TTA CCT CCT TGT ACA CAC AGG TTG TTC
────┘ └──────────────────────────────────────────────────────────
```

oligo 5

```
┐
T
A
┘
```

The plasmid pDJB117 was partially digested with *Sca*I and linearised DNA was isolated from an agarose gel and then digested with *Bam*HI. This DNA was ligated with linker 6 to form pDJB118 (Figure 9).

The *Hin*dIII fragment from pDJB118 (Figure 9) containing the uPA-PAI-2 coding sequence was then intr duced into the expression plasmid pDBP6 to form pDBUP1 (Figure 10). Plasmid pDBUP1 contains a *PRB1* promoter fragment to control transcription of the uPA-PAI-2 sequence and the *ADH1* transcription terminator. It also contains sequences of the bacterial plasmid pAT153 (Twigg and Sherratt, 1980), the *leu2d* gene for selection of transformants and part of the 2μm plasmid to provide replication function in *S. cerevisiae*. The plasmid was introduced into a suitable *S. cerevisiae* strain wherein it effected production of the hybrid protein as a soluble intracellular protein which was recognised by anti-PAI-2 and anti-uPA antibodies in Western blotting experiments.

The protein was purified from cell lysates by copper chelate and anion exchange chromatography and was found to inhibit uPA with similar kinetics to PAI-2 itself, ie second order inhibition rate constant of 1.8×10^6 $M^{-1}S^{-1}$, determined according to Thorsen *et al* (1988).

The uPA growth factor domain was shown to be functional by its ability to mediate specific binding to U937 cells. The cells were first treated with 50mM glycine HCl, 0.1M NaCl, pH3.0 for 3 minutes at 4°C to remove endogenous cell-bound uPA. The cell suspension was neutralised by the addition of one-fifth volume of 0.5M Hepes, 0.1M NaCl, pH7.5 and then the cells were washed twice with PBS (Dulbecco's Ca^{2+} and Mg^{2+} free ph sphate buffered saline) containing 0.1% (w/v) bovine serum albumin (PBS + BSA).

Aliquots of cells (0.5×10^6) were then incubated with or without approximately 0.5μg uPA-PAI-2 hybrid protein in PBS + BSA for 90 min on ice and then for a further 60 min with 10% (v/v) pooled normal human serum in Dulbecco's PBS with 20mM glucose, 5mM Na_2EDTA, 0.1% (w/v) BSA, 0.1% (w/v) NaN_3 (PBS + NHS) to block cell membrane F_c receptors. The cells were washed three times with PBS + NHS and then incubated with goat anti-PAI-2 antibody (American Diagnostica Inc) in PBS + NHS for 30 min on ice. The cells were then washed twice with PBS + NHS and then incubated with anti-goat IgG antibody conjugated to fluorescein isothiocyanate in PBS + NHS for 30 min on ice. Finally the cells were washed twice with PBS + NHS and resuspended in 100μl PBS + NHS. The cells were analysed (10,000 cells per analysis) with an Epics 541 Flow Cytometer using a 488nm laser light source and green light fluorescence detection. Figure 11 is an example of the data obtained from such an experiment and shows and increase in fluorescence bound to the cells incubated with the uPA-PAI-2 hybrid indicating that the protein binds to U937 cells. Additional experiments showed that PAI-2 itself did not bind to cells appreciably and that the binding of uPA-PAI-2 to U937 cells was competed by pro-uPA and could be inhibited by anti-uPA antibody but not by anti-PAI-2 antibody. These data demonstrated that the uPA-PAI-2 hybrid protein was able to bind to cells via the uPA receptor. Both the uPA growth factor domain and the PAI-2 domain of the hybrid protein were thus demonstrated to be functional.

Example 2

A human α_1-antitrypsin cDNA, identical in sequence to that described in the Genbank database, release 60, accession number X01683 V00496, locus HUMA1ATR was amplified by Polymerase Chain Reaction using a Perkin Elmer Cetus DNA thermal cycler according to the manufacturer's instructions. The DNA sequence of the two primary oligonucleotides were:

5' Oligonucleotide

5'-GAGGATCCCCAGGGAGATGCTGCCCAGAAG-3'

3' Oligonucleotide

5'-GGGGAAGCTTTTATTTTTGGGTGGGATTCACCACTTTTCC-3'

Upon amplification both the 5' and 3' termini of the α_1-antitrypsin sequence became modified as follows:

5' Terminus

original

G GTC CCT GTC TCC CTG GCT GAG GAT CCC CAG GGA GAT GCT GCC CAG

V P V S L A E D P Q G D A A Q

BamHI

AAG

K

modified

GAG GAT CCC CAG GGA GAT GCT GCC CAG AAG

E D P Q G D A A Q K

BamHI

3' Terminus

original GGA AAA GTG GTG AAT CCC ACC CAA AAA TAA CTGCCTCTCG

G K V V N P T Q K *

modified GGA AAA GTG GTG AAT CCC ACC CAA AAA TAA AAGCTTCCCC

G K V V N P T Q K * *HindIII*

These modifications remove the 23 amino acid signal sequence and introduce a *Hin*dIII restriction site at the 3' end of the cDNA.

The 1.22kbp modified cDNA was purified, digested with *Hin*dIII and *Bam*HI inserted and cloned into the *Hin*dIII-*Bam*HI sites of M13mp19 (Yanisch-Perron *et al* (1985) *Gene* **33**, 103-119) generating pDBA1 (Figure 12). The integrity of human α_1-antitrypsin was confirmed by dideoxynucleotide sequencing.

A sequence encoding α_1-antitrypsin Pittsburgh (α_1AT-P) (Owen *et al*, 1983) was created using oligonucleotide-directed mutagenesis of pDBA1 using the oligonucleotide shown below

5'-GAGGCCATACCCAGGTCTATCCCC-3'

This resulted in a change in the codon for methionine 358 such that it coded instead for an arginine ie ATG -> AGG. This plasmid is pDBA2 (Fig 12).

Two oligonucleotides were synthesised and then annealed to form a linker (Linker 7) to encode the C-terminal portion of the uPA growth factor domain (amino acids 24-47) and the start of the α_1AT-P sequence.

Linker 7

```
     Y   F   S   N   I   H   W   C   N   C   P   K   K   F
   AGTAC TTC TCC AAC ATT CAC TGG TGC AAC TGC CCA AAG AAA TTC
CATGTCATG AAG AGG TTG TAA GTG ACC ACG TTG ACG GGT TTC TTT AAG
     └─────┘
     ScaI

 G   G   Q   H   C   E   I   D   K   S   E
GGA GGG CAG CAC TGT GAA ATA GAT AAG TCA GAG
CCT CCC GTC GTG ACA CTT TAT CTA TTC AGT CTCCTAG
                                    └─────┘
                                    BamHI
```

This linker was ligated with *Kpn*I-*Bam*HI digested double stranded pDBA2 to form pDBUA1 (Fig 12).

A second linker representing the *PRB1* ATG environment, a methionine initiation codon and DNA encoding the N-terminal portion of the uPA growth domain (amino acids 1-23) was assembled by annealing the four oligonucleotides shown below:-

Linker 8

oligo 7

```
                                        M   S   N   E   L   H   Q   V
AATTAAGCTTAACCTAATTCTAACAAGCAAAG ATG AGC AAT GAA CTT CAT CAG GTA
    TTCGAATTGGATTAAGATTGTTCGTTTC TAC TCG TTA CTT GAA GTA GTC CAT
    ┌────┘
    HindIII
    └──────────────────────────────────────────────────────────────
```

oligo 6

oligo 4

```
 P   S   N   C   D   C   L   N   G   G   T   C   V   S   N   K
CCA TCG AAC TGT GAC TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG
GGT AGC TTG ACA CTG ACA GAT TTA CCT CCT TGT ACA CAC AGG TTG TTC
────┘ └──────────────────────────────────────────────────────────
```

oligo 5

T

A

Double stranded pDBUA1 was digested with *Sca*I and *Eco*RI and then ligated with linker 8 to form pDBUA2 (Fig 12). The *Hin*dIII fragment of pDBUA2 was then introduced into the expression plasmid pDBP6 to form pDBUA3 (Fig 12). This plasmid was then introduced into a suitable *S. cerevisiae* strain wherein it effected production of the hybrid protein which was recognised by anti-α_1AT and anti-uPA antibodies in Western blotting experiments.

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CLAIMS

1. A molecule comprising a first region which is capable of binding to a tumour cell and a second region which is capable of inhibiting a first protease.

2. A molecule according to Claim 1 wherein the first region binds to a receptor for a second, tumour-associated protease which may be the same as or different from the first protease.

3. A molecule according to Claim 2 wherein the second tumour-associated protease is uPA.

4. A molecule according to Claim 2 or 3 wherein the first region binds to a receptor in such a way as to block binding thereto by the second protease.

5. A molecule according to Claim 4 wherein the first region comprises a receptor-binding portion of the second protease or a receptor-binding variant of the second protease.

6. A molecule according to any one of the preceding claims wherein the first protease is thrombin.

7. A molecule according to any one of Claims 1 to 5 wherein the first protease is tum ur-associated.

8. A molecule according to Claim 7 wherein the first protease is uPA, plasmin, stromelysin or cathepsin.

9. A molecule according to Claim 8 wherein the first region comprises a peptide corresponding to amino acids 20-30 of mature uPA.

10. A molecule according to Claim 9 wherein the first region comprises a peptide corresponding to amino acids 12 to 32 of mature uPA.

11. A molecule according to Claim 10 wherein the first region comprises a peptide corresponding to amino acids 1 to 47 of mature uPA.

12. A molecule according to Claim 10 or 11 wherein the first region comprises a non-plasminogen-activating mutant of uPA or pro-uPA.

13. A molecule according to any one of Claims 9 to 12 wherein the second region is PAI-2 or a uPA-inhibiting variant or fragment thereof.

14. A molecule according to any one of Claims 9 to 12 wherein the second region is α_1-antitrypsin (Pittsburgh) or a uPA-inhibiting or plasmin-inhibiting variant or fragment thereof.

15. A molecule according to any one of the preceding claims comprising a polypeptide consisting of the first and second regions and, optionally, an intervening amino acid sequence combined to form a single, linear amino acid sequence.

16. A nucleotide sequence encoding a polypeptide as defined in Claim 15.

17. A process of producing a molecule according to any one of Claims 1 to 15 comprising (a) preparing the said first and second regions and joining them together or (b) preparing the first and second regions and, optionally, an intervening amino acid sequence, as a single linear polypeptide by expressing a nucleotide sequence encoding the polypeptide in a suitable host cell transformed with the nucleotide sequence.

18. A process according to Claim 17 (b) wherein the host cell is a *Saccharomyces cerevisiae* cell.

19. A method of combatting neoplasms comprising administering to a patient a molecule according to any one of Claims 1 to 15.

20. A compound which binds specifically to uPAR and to PAI-2.

21. A compound according to Claim 20 wherein the compound is a bispecific antibody.

22. A pharmaceutical composition comprising a molecule according to any one of Claims 1 to 15 or a compound according to Claim 20 or 21.

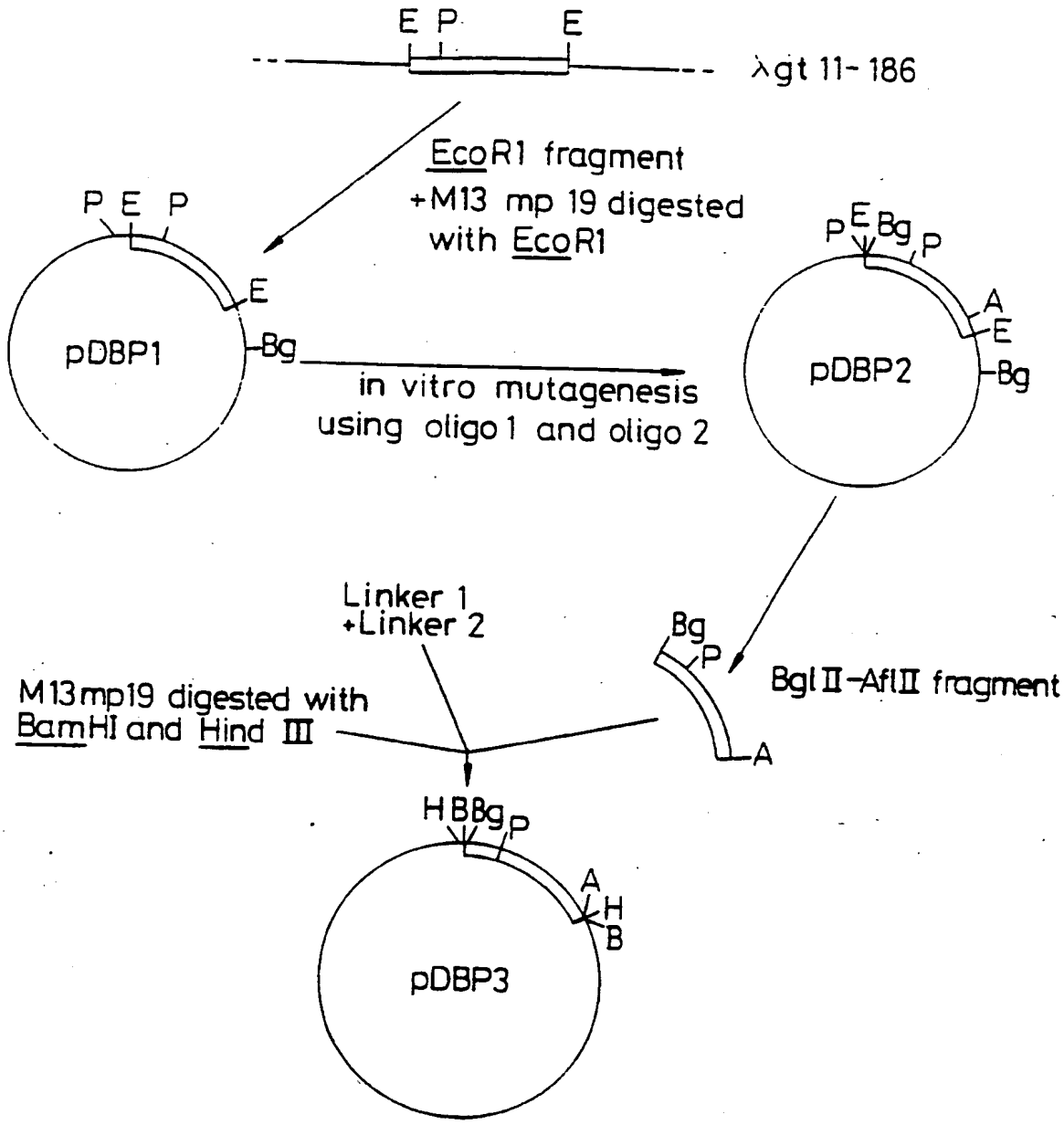
E P E
λgt 11-186
EcoR1 fragment
+M13 mp 19 digested
with EcoR1
P E P
E
pDBP1
Bg
in vitro mutagenesis
using oligo 1 and oligo 2
P E Bg P
A
E
pDBP2
Bg
Linker 1
+Linker 2
M13mp19 digested with
BamHI and Hind III
Bg
P
BglII-AflII fragment
A
H B Bg P
A
H
B
pDBP3

Fig. 1

```
AGCAACTCAG AGAATAACCA GAGAACAACC AGATTGAAAC A ATG GAG GAT CTT
                                          53
                                                  Met Glu Asp Leu
                                                    1

TGT GTG GCA AAC ACA CTC TTT GCC CTC AAT TTA TTC AAG CAT CTG GCA
                                                 101
Cys Val Ala Asn Thr Leu Phe Ala Leu Asn Leu Phe Lys His Leu Ala
  5                  10                  15                  20

AAA GCA AGC CCC ACC CAG AAC CTC TTC CTC TCC CCA TGG AGC ATC TCG
                                                 149
Lys Ala Ser Pro Thr Gln Asn Leu Phe Leu Ser Pro Trp Ser Ile Ser
                  25                  30                  35

TCC ACC ATG GCC ATG GTC TAC ATG GGC TCC AGG GGC AGC ACC GAA GAC
                                                 197
Ser Thr Met Ala Met Val Tyr Met Gly Ser Arg Gly Ser Thr Glu Asp
            40                  45                  50

CAG ATG GCC AAG GTG CTT CAG TTT AAT GAA GTG GGA GCC AAT GCA GTT
                                                 245
Gln Met Ala Lys Val Leu Gln Phe Asn Glu Val Gly Ala Asn Ala Val
        55                  60                  65

ACC CCC ATG ACT CCA GAG AAC TTT ACC AGC TGT GGG TTC ATG CAG CAG
                                                 293
Thr Pro Met Thr Pro Glu Asn Phe Thr Ser Cys Gly Phe Met Gln Gln
    70                  75                  80

ATC CAG AAG GGT AGT TAT CCT GAT GCG ATT TTG CAG GCA CAA GCT GCA
                                                 341
Ile Gln Lys Gly Ser Tyr Pro Asp Ala Ile Leu Gln Ala Gln Ala Ala
 85                  90                  95                  100

GAT AAA ATC CAT TCA TCC TTC CGC TCT CTC AGC TCT GCA ATC AAT GCA
                                                 389
Asp Lys Ile His Ser Ser Phe Arg Ser Leu Ser Ser Ala Ile Asn Ala
                105                 110                 115
```

Figure 2 (first sheet)

```
TCC ACA GGG AAT TAT TTA CTG GAA AGT GTC AAT AAG CTG TTT GGT GAG
    437
Ser Thr Gly Asn Tyr Leu Leu Glu Ser Val Asn Lys Leu Phe Gly Glu
          120                 125                 130

AAG TCT GCG AGC TTC CGG GAA GAA TAT ATT CGA CTC TGT CAG AAA TAT
    485
Lys Ser Ala Ser Phe Arg Glu Glu Tyr Ile Arg Leu Cys Gln Lys Tyr
      135                 140                 145

TAC TCC TCA GAA CCC CAG GCA GTA GAC TTC CTA GAA TGT GCA GAA GAA
    533
Tyr Ser Ser Glu Pro Gln Ala Val Asp Phe Leu Glu Cys Ala Glu Glu
  150                 155                 160

GCT AGA AAA AAG ATT AAT TCC TGG GTC AAG ACT CAA ACC AAA GGC AAA
    581
Ala Arg Lys Lys Ile Asn Ser Trp Val Lys Thr Gln Thr Lys Gly Lys
165                 170                 175                 180

ATC CCA AAC TTG TTA CCT GAA GGT TCT GTA GAT GGG GAT ACC AGG ATG
    629
Ile Pro Asn Leu Leu Pro Glu Gly Ser Val Asp Gly Asp Thr Arg Met
                185                 190                 195

GTC CTG GTG AAT GCT GTC TAC TTC AAA GGA AAG TGG AAA ACT CCA TTT
    677
Val Leu Val Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys Thr Pro Phe
          200                 205                 210

GAG AAG AAA CTA AAT GGG CTT TAT CCT TTC CGT GTA AAC TCG GCT CAG
    725
Glu Lys Lys Leu Asn Gly Leu Tyr Pro Phe Arg Val Asn Ser Ala Gln
      215                 220                 225

CGC ACA CCT GTA CAG ATG ATG TAC TTG CGT GAA AAG CTA AAC ATT GGA
    773
Arg Thr Pro Val Gln Met Met Tyr Leu Arg Glu Lys Leu Asn Ile Gly
  230                 235                 240
```

Figure 2 (second sheet)

```
TAC ATA GAA GAC CTA AAG GCT CAG ATT CTA GAA CTC CCA TAT GCT GGA
     821
Tyr Ile Glu Asp Leu Lys Ala Gln Ile Leu Glu Leu Pro Tyr Ala Gly
245                 250                 255                 260

GAT GTT AGC ATG TTC TTG TTG CTT CCA GAT GAA ATT GCC GAT GTG TCC
     869
Asp Val Ser Met Phe Leu Leu Leu Pro Asp Glu Ile Ala Asp Val Ser
                265                 270                 275

ACT GGC TTG GAG CTG CTG GAA AGT GAA ATA ACC TAT GAC AAA CTC AAC
     917
Thr Gly Leu Glu Leu Leu Glu Ser Glu Ile Thr Tyr Asp Lys Leu Asn
            280                 285                 290

AAG TGG ACC AGC AAA GAC AAA ATG GCT GAA GAT GAA GTT GAG GTA TAC
     965
Lys Trp Thr Ser Lys Asp Lys Met Ala Glu Asp Glu Val Glu Val Tyr
        295                 300                 305

ATA CCC CAG TTC AAA TTA GAA GAG CAT TAT GAA CTC AGA TCC ATT CTG
    1013
Ile Pro Gln Phe Lys Leu Glu Glu His Tyr Glu Leu Arg Ser Ile Leu
    310                 315                 320

AGA AGC ATG GGC ATG GAG GAC GCC TTC AAC AAG GGA CGG GCC AAT TTC
    1061
Arg Ser Met Gly Met Glu Asp Ala Phe Asn Lys Gly Arg Ala Asn Phe
325                 330                 335                 340

TCA GGG ATG TCG GAG AGG AAT GAC CTG TTT CTT TCT GAA GTG TTC CAC
    1109
Ser Gly Met Ser Glu Arg Asn Asp Leu Phe Leu Ser Glu Val Phe His
                345                 350                 355

CAA GCC ATG GTG GAT GTG AAT GAG GAG GGC ACT GAA GCA GCC GCT GGC
    1157
Gln Ala Met Val Asp Val Asn Glu Glu Gly Thr Glu Ala Ala Ala Gly
            360                 365                 370
```

Figure 2 (third sheet)

```
ACA GGA GGT GTT ATG ACA GGG AGA ACT GGA CAT GGA GGC CCA CAG TTT
    1205
Thr Gly Gly Val Met Thr Gly Arg Thr Gly His Gly Gly Pro Gln Phe
        375                 380                 385

GTG GCA GAT CAT CCT TTT CTT TTT CTT ATT ATG CAT AAG ATA ACC AAC
    1253
Val Ala Asp His Pro Phe Leu Phe Leu Ile Met His Lys Ile Thr Asn
    390                 395                 400

TGC ATT TTA TTT TTC GGC AGA TTT TCC TCA CCC TAAAACTAAG CGTGCTGCTT
  1306
Cys Ile Leu Phe Phe Gly Arg Phe Ser Ser Pro
405                 410                 415

CTGCAAAAGA TTTTTGTAGA TGAGCTGTGT GCCTCAGAAT TGCTATTTCA AATTGCCAAA
  1366

AATTTAGAGA TGTTTTCTAC ATATTTCTGC TCTTCTGAAC AACTTC
    1412
```

Figure 2 (fourth sheet)

*Hin*dIII

aagcttactgctgacctttgccttggtccttgtacttgtctcgattgctgnnaccattaa
cggtttttttgttcttacttaatctttttttttcccgacgacgcaaaatgtcagaaaagata
taaanagagcaacgtttcctgctacatgaaacacgctggcatatagaagaacttcaacga
gcgaggctctctgaacaatctgctaatcgtgcaagtaagggttctcgtgacagttcaccg
tgtataaacaagnnnaaacagataaacagtcagatatgcttaatttttaactgtaggctg
tgccgacatgccattttatgcggctcacttctaacaaaagtgaccatgacgcacaagcaa
gcaaacagccaagtaaggaagcagacagcttcacagcacacacacaccgttgtgtacgaa

*Sna*BI

aatcctctgcagaagagtgtatatgccactcatccacacttccgcagcgcagtacgtaat
gcggtatcgtgaaagcgaaaaaaaaactaacagtagataagacagatagacagatagaga
tggacgagaaacaggggggggagaaaaggggaaaagagaaggaaagaaagactcatctatc
gcagataagacaatcaaccctcatggcgcctccaaccaccatccgcactagggaccaagc
gctcgcaccgttagcaacgcttgactcacaaaccaactgccggctgaaagagcttgtgca
atgggagtgccaattcaaaggagccgaatacgtctgctcgccttttaagaggcttttga
acactgcattgcacccgacaaatcagccactaactacgaggtcacggacacatataccaa
tagttaaaaattacatatactctatatagcacagtagtgtgataaataaaaaattttgcc
aagacttttttaaactgcacccgacagatcaggtctgtgcctactatgcacttatgcccg
gggtcccgggaggagaaaaaacgagggctgggaaatgtccgtggactttaaacgctccgg
gttagcagagtagcagggctttcggctttggaaatttaggtgacttgttgaaaaagcaaa
atttgggctcagtaatgccactgcagtggcttatcacgccaggactgcgggagtggcggg
ggcaaacacacccgcgataaagagcgcgatgaatataaaaggggccaatgttacgtccc
gttatattggagttcttcccatacaaacttaagagtccaattagcttcatcgccaataaa
aaaacaaactaaacctaattctaacaagcaaagatgaagttagaaaatactctatttaca
ctcggtgccctagggagcatctctgctgctttggtcatcccaaatcttgaaaatgccgcc

*Eco*RI

gaccaccacgaactgattaacaaggaagatcaccacgagagacccagaaaagtggaattc

Figure 3

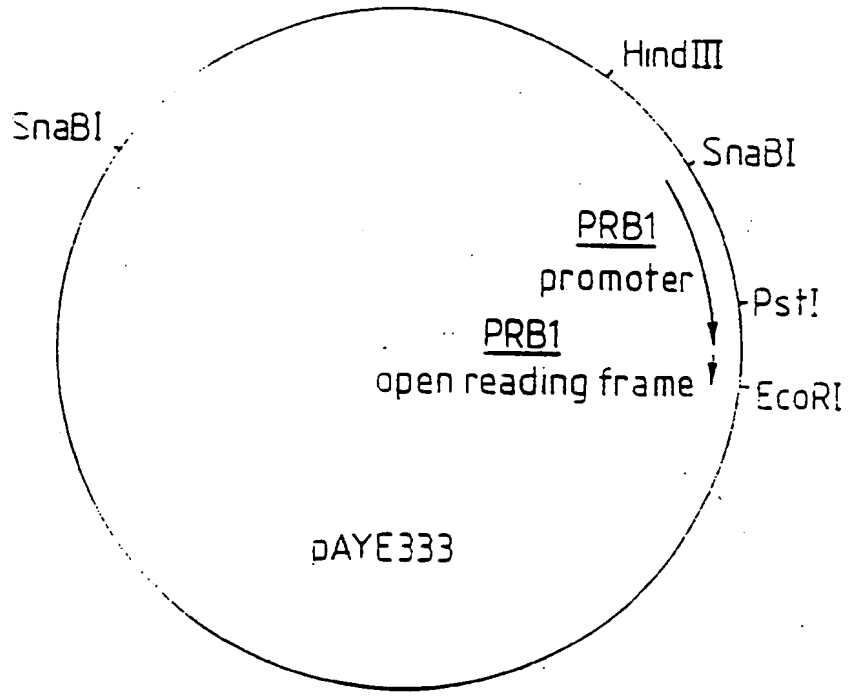
HindIII
SnaBI
SnaBI
PRB1
promoter
PstI
PRB1
open reading frame
EcoRI
pAYE333

FIGURE 4

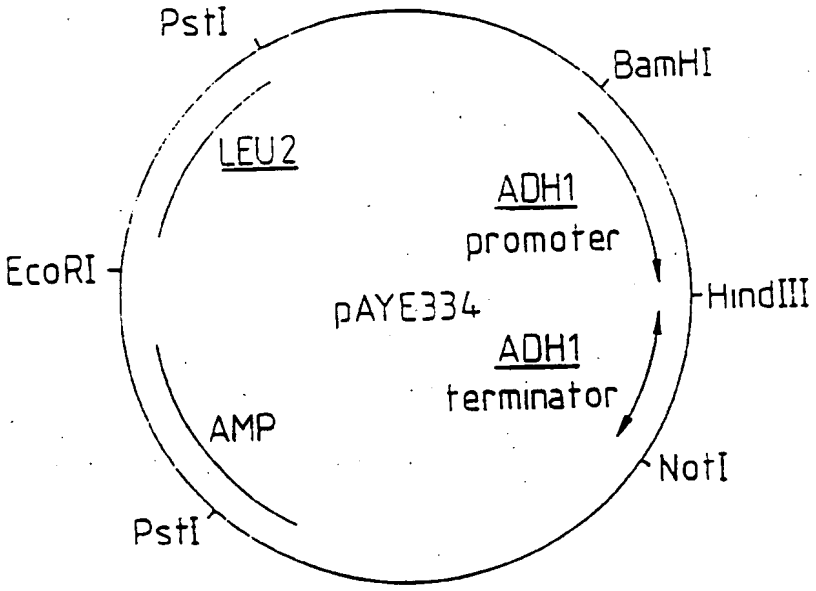
PstI
BamHI
LEU2
ADH1
promoter
EcoRI
pAYE334
HindIII
ADH1
terminator
AMP
NotI
PstI

FIGURE 5

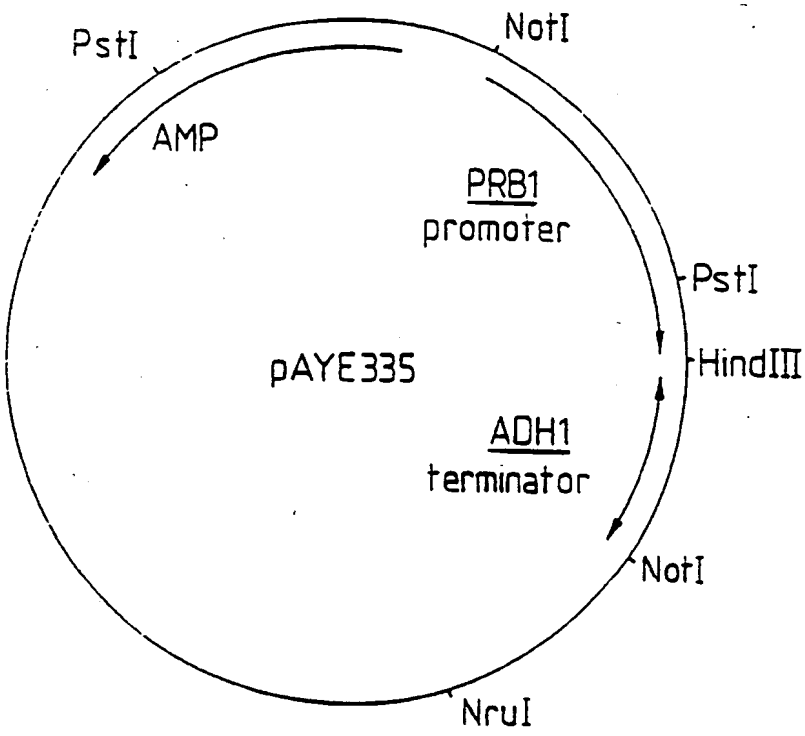
PstI
NotI
AMP
PRB1
promoter
PstI
HindIII
pAYE335
ADH1
terminator
NotI
NruI

FIGURE 6

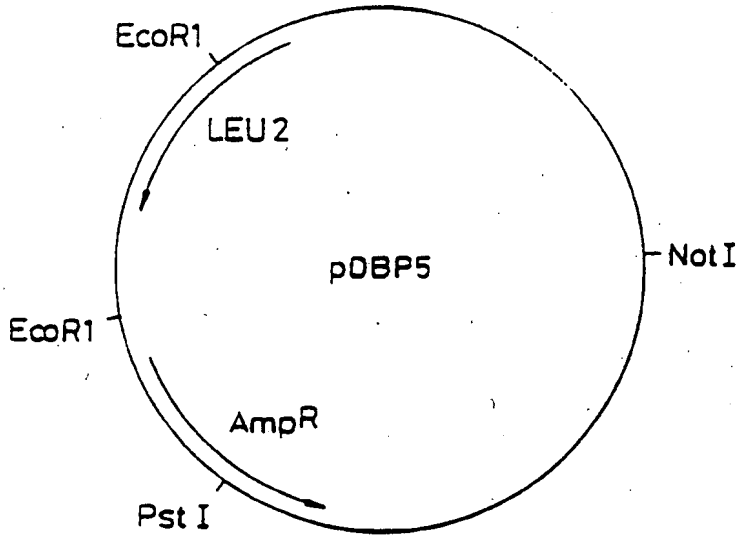

FIGURE 7

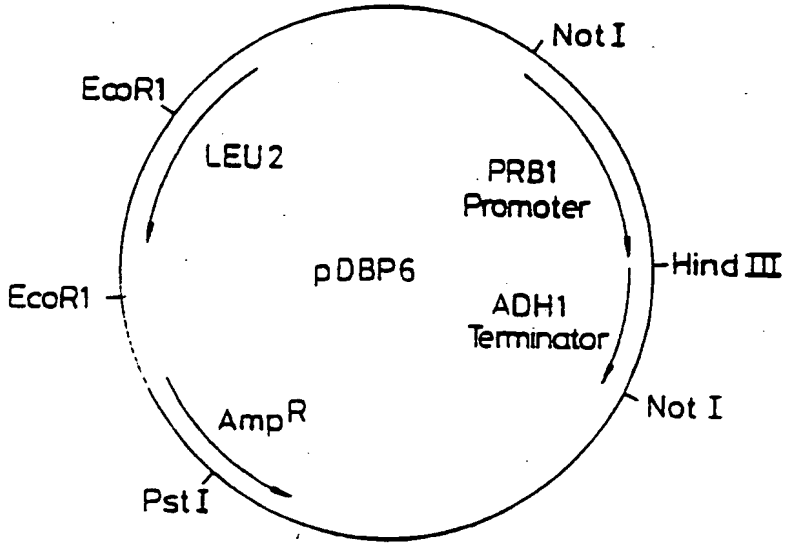
Not I
EcoR1
LEU2
PRB1
Promoter
pDBP6
Hind III
EcoR1
ADH1
Terminator
Not I
AmpR
Pst I

FIGURE 8

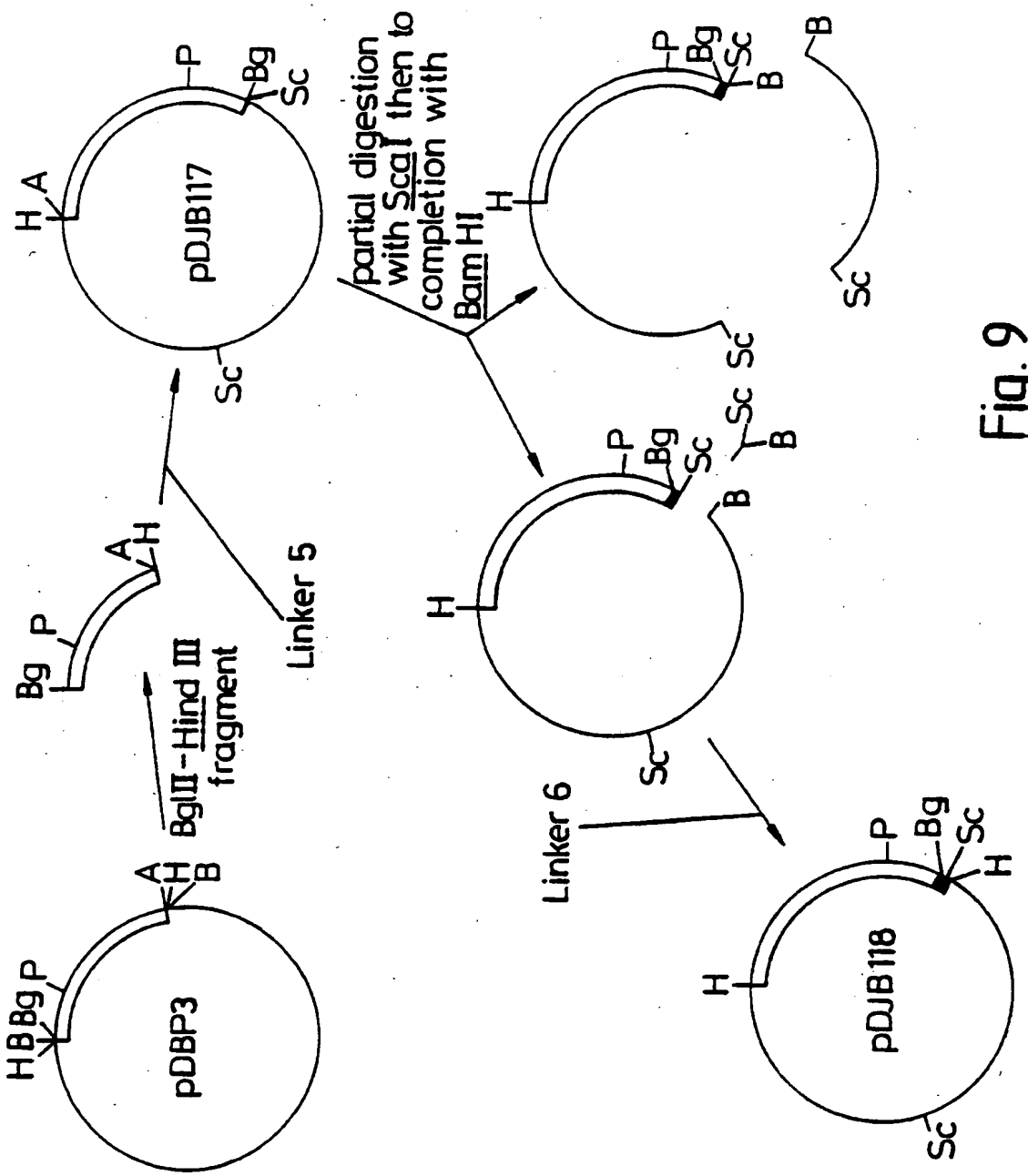
pDJB117
pDBP3
pDJB118
BglII-HindIII fragment
Linker 5
Linker 6
partial digestion with ScaI then to completion with BamHI
P
Bg
Sc
H
A
B

Fig. 9

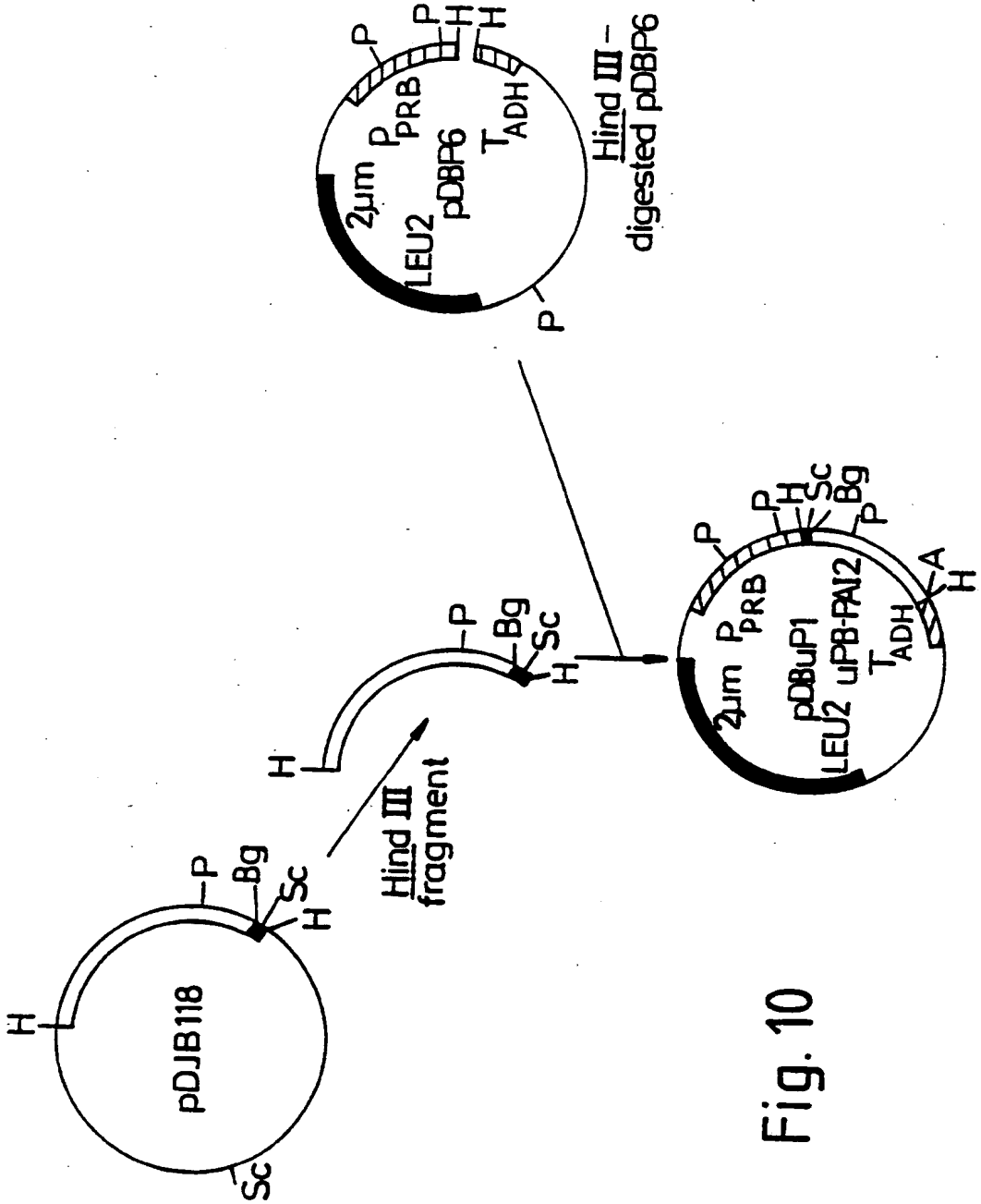
Hind III – digested pDBP6
2µm
LEU2
pDBP6
P
H
Bg
Sc
A
Hind III fragment
pDJB118
pDBuP1
uPB-PAI2

Fig. 10

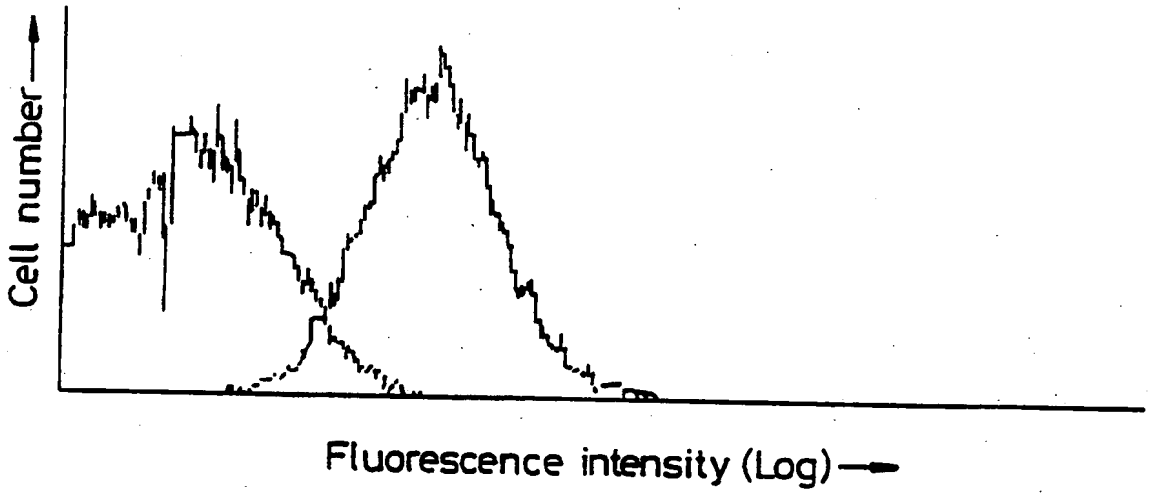

Fig. 11

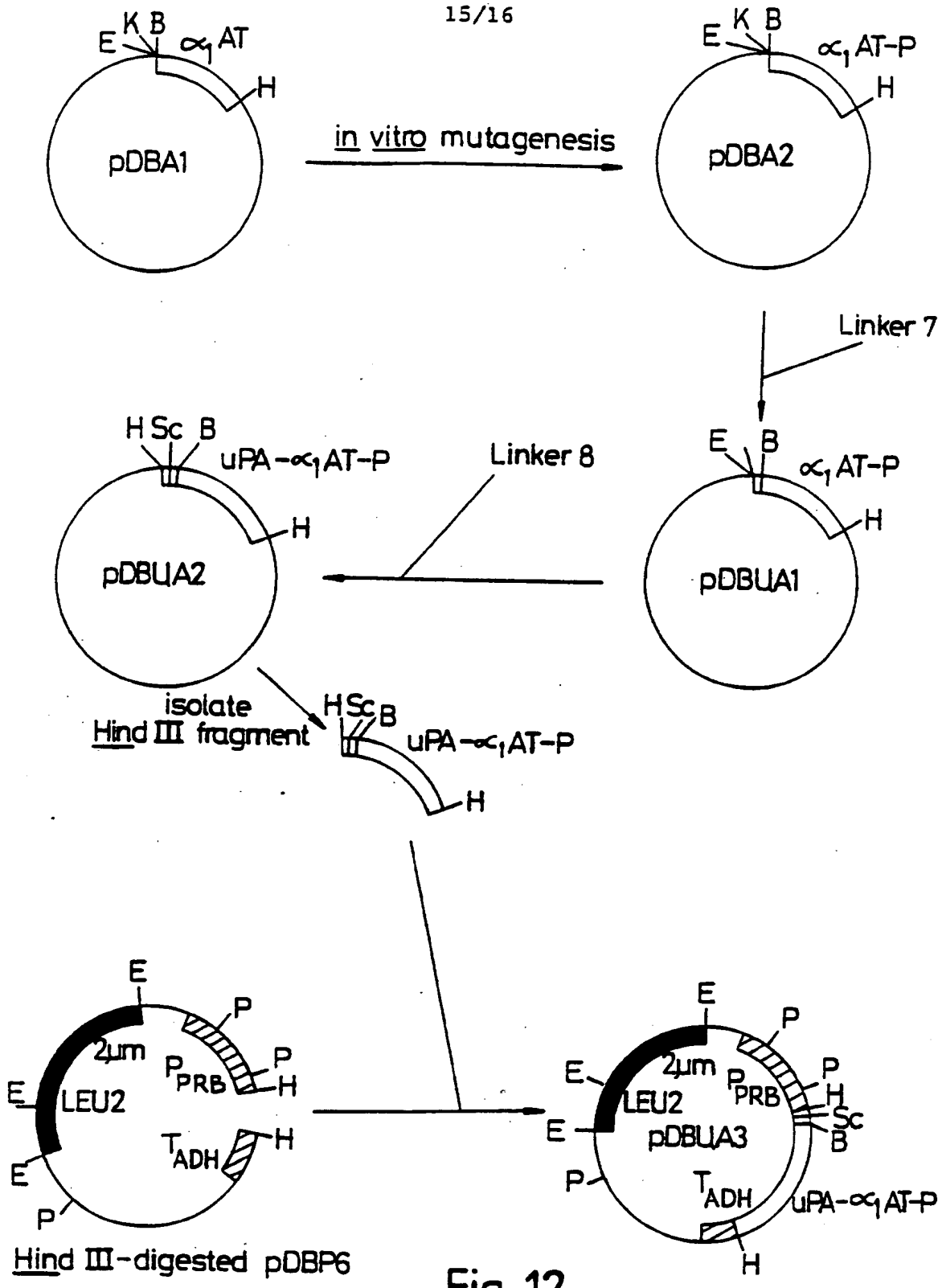
E K B
α_1AT
H
pDBA1
in vitro mutagenesis
E K B
α_1AT-P
H
pDBA2
Linker 7
H Sc B
uPA-α_1AT-P
H
pDBUA2
Linker 8
E B
α_1AT-P
H
pDBUA1
isolate
Hind III fragment
H Sc B
uPA-α_1AT-P
H
E
P
2µm
P_{PRB}
P
H
E
LEU2
T_{ADH}
H
E
P
Hind III-digested pDBP6
E
P
2µm
E
LEU2
P_{PRB}
P
H
Sc
B
E
pDBUA3
P
T_{ADH}
uPA-α_1AT-P
H

Fig. 12

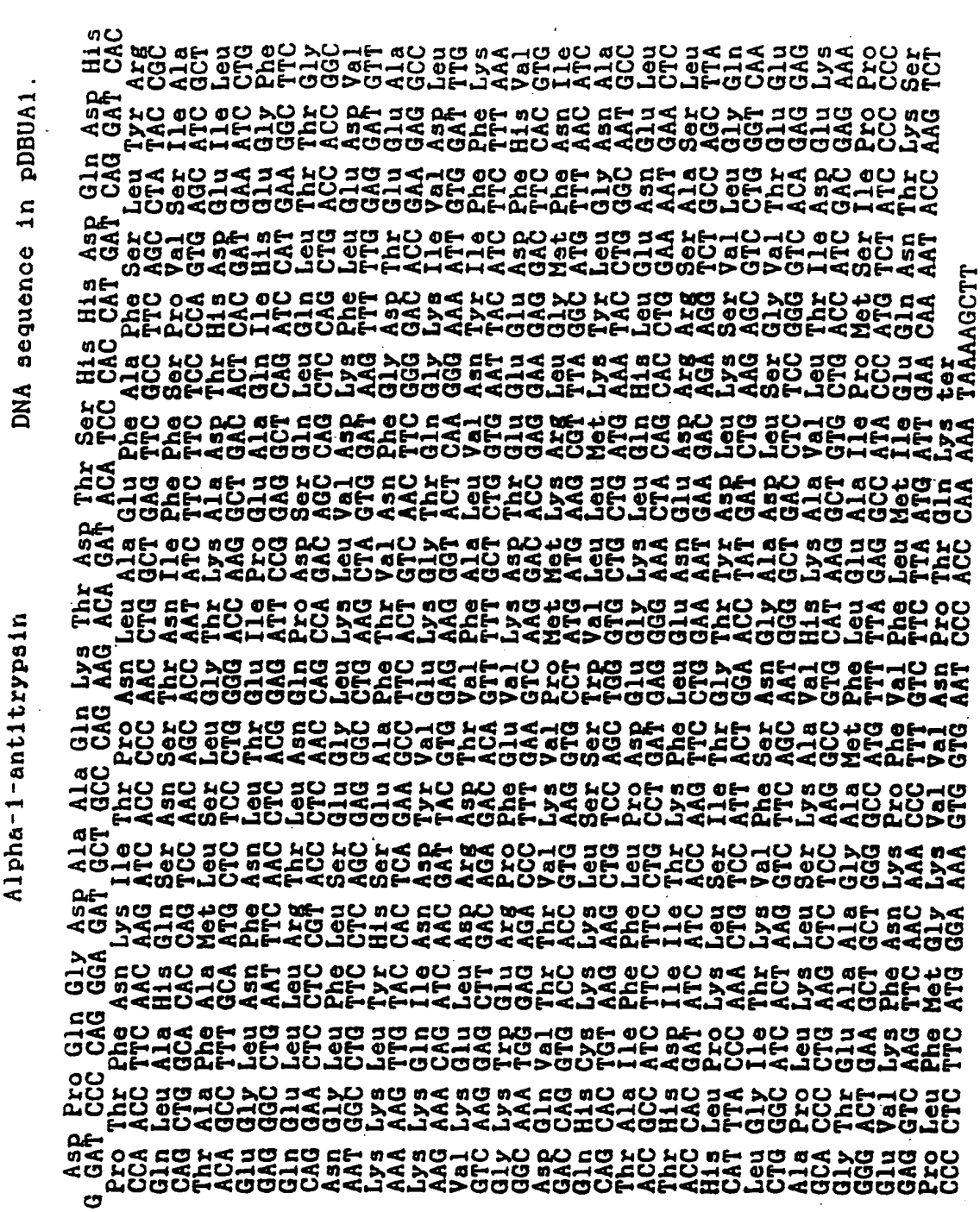

FIGURE 13

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01322

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/00, A 61 K 39/395, 47/48, C 12 N 15/62
C 12 P 21/02, 08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	WO, A1, 9012091 (CANCERFORSKNINGSFONDET AF) 18 October 1990, see pages 1-26,40,70, in particular page 24, lines 3-5, examples 8-9 and fig 29-30 as well as claims 52-58,62,73-77	1-4,7,8, 15-17
X	J. NATL.CANCER INST., vol. 74, No. 5, 1985, Jung-Yaw Lin et al: "Antitumor Lectin-Trypsin Inhibitor Conjugate ", see page 1031 - page 1035 especially "Discussion".	1,6-8
X	THE FASEB JOURNAL, vol. 4, No. 7, 1990, A.J. Wolfson et al: "Insertion of a protease-binding loop into interleukin-1B ", page A1762	1,6-8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th November 1991	21. 11. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	BIOCHEMISTRY INTERNATIONAL, vol. 19, No. 2, 1989, Jung-Yaw Lin et al: "CHIMERIC PROTEIN: ABRIN B CHAIN-TRYPSIN INHIBITOR CONJUGATE AS A NEW ANTITUMOR AGENT ", see page 313 - page 323 see in particular "Discussion"	1,6-8
Y		2,3,15-17,20, 21
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O,Y	THROMBOSIS RESEARCH Supplement XI, 1990, M T Masucci: "The receptor for human urokinase: a potential target for anti-invasive and anti-metastatic therapy ", International symposium on biochemical and pharmacological aspects of thrombotic processes (Brescia ITA), see page 49 - page 60	2,3,15-17,20-21
	--	
A	Chemical Abstracts, volume 88, no. 23, 5 June 1978, (Columbus, Ohio, US), Verloes,Rene et al: "Tumor growth inhibition mediated by trypsin inhibitor or urokinase inhibitors.", see page 39, abstract 163955w, & Eur.J.Cancer 1978, 14(1), 23- 31	1
	--	
A	EP, A1, 0301122 (MAEDA, HIROSHI) 1 February 1989, see the whole document	1,6,7
	--	
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, No. 5, 1988, Lars S. Nielsen et al: "A55,000-60,000 M Receptor Protein for Urokinase-type Plasminogen Activator ", see page 2358 - page 2363 especially page 2358, right column and page 2363	1-5
	--	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	WO, A1, 9100912 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 24 January 1991, see page 15 - page 20	1,15-17

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/GB 91/01322

SA 50125

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 27/09/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9012091	18/10/90	AU-D- 5528090	05/11/90
EP-A1- 0301122	01/02/89	JP-A- 62294622	22/12/87
WO-A1- 9100912	24/01/91	NONE	